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14. ABSTRACT

The purpose of this proposal was to investigate the response of prostate cancer to a series of camptothecin analogs, which are drugs that target DNA topoisomerase I. This final report summarizes the accomplishments toward the original goals of the project. We note that while some experimental problems were encountered that limited the completion of all tasks, good progress was made overall on the proposed research.

15. SUBJECT TERMS
topoisomerase I; nucleolus; RNA synthesis; transcription; DNA damage, Drug resistance

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INTRODUCTION:

This final report for DAMD17-02-1-0078 summarizes the accomplishments toward the tasks outlined in the original proposal for this grant. The goal of the project was to try to understand why, despite the availability of topoisomerase I in prostate cancer, chemotherapeutic agents that target this enzyme are ineffective in treating prostate cancer. Members of the camptothecin family that specifically target topo I were studied in prostate cancer cell lines grown *in vitro*. The project was meant to attack this problem from three separate directions using three assays. The first assay was that of initial topo I distribution in normal and cancer cells and its response to CPT analog treatment. The second assay was for selection of CPT analogs that form slowly-reversing topo I-DNA complexes. The third was measurement of the onset of apoptosis in prostate cells treated with the different analogs. This final report documents the work performed throughout the lifetime of the funded project.

BODY:

Tasks from Approved Statement of Work

Task 1: Measuring Topoisomerase I distribution in prostate cancer and its relationship to response in camptothecins (Months 1-12):

a. Optimize immunostaining for 6 prostate cancer cell lines being used.(Months 1-4).
b. Grow normal prostate lines in culture (months 5-8)
c. Analyze redistribution of topoisomerase I in response to camptothecin analogs (months 8-12).

Work toward Task 1.

Year 1: At the time of submission of our original proposal, our work with transfection of TSU-Pr1with an EGFP-topo I chimera appeared to have good success in producing prostate cancer cells with transient expression of this fluorescent protein (see below). Consequently, work on the immunostaining of cells (Task 1) was postponed to concentrate fully on Task 2.

Years 2 & 3: we attempted to develop PC-3 lines that were stably transfected with a fluorescent topoisomerase I, but due to the complex nature of topo I expression, we had to return to immunostaining. This provided less than satisfactory results for completing task 2. Using rabbit immunoserum meant that the reactivity of the serum varied from lot to lot, and hence we obtained apparent non-specific immunostaining for topo I.

Year 4: We devoted an entire year of attempting to complete immunostaining of PC-3 cells with rabbit serum obtained from colleagues at St. Jude Children's Research Hospital. An undergraduate in the lab, Natalie Young, tried several different fixation and staining protocols, but was unable to optimize staining. Hence, we were unable to complete Task 1 as described. Our conclusion is that the delicate nature of the nucleolus of prostate cancer cells does not allow it to maintain the structural integrity of other epithelial cells treated with the same conditions. More gentle staining protocols need to be developed.

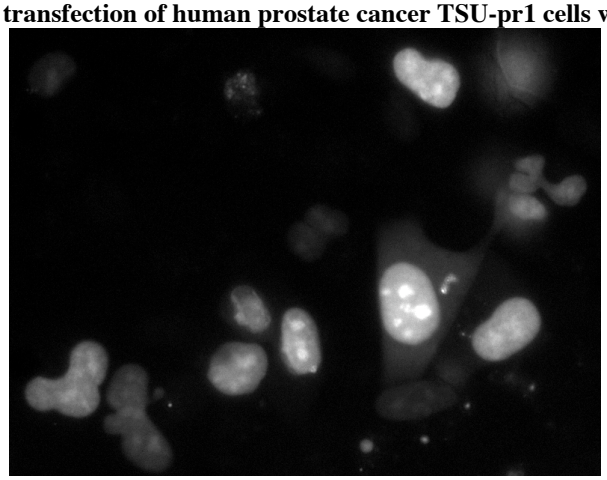
Task 2. Develop prostate cell lines expressing topo I-EGFP fusion protein (Months 12-18).

a. Select transfectants of all 6 prostate cancer lines expressing high topo I-EGFP levels (months 12-15).
b. prepare vectors for knockout/knockin of topo I-EGFP (months 16-18).

Work toward Task 2.

Year 1: As noted in the original proposal, immunostaining does not let us generate continuous distribution data for topo I, since we must harvest cells at selected time points. A more intriguing way of following topo I distribution with time was to use cells with a transfected topo I-enhanced green fluorescent protein conjugate. A plasmid derived from pEGFP-C1 (CLONTECH) containing the human topo I-EGFP protein was provided to us by Dr. Eric Rubin, Robert Wood Johnson School of Medicine. Fig. 1 shows a transient transfection of TSU-pr1 prostate cancer cells with this plasmid.

Fig. 1. Transient transfection of human prostate cancer TSU-pr1 cells with topo I-EGFP.



We originally transfected TSU-pr1, and subsequently PC-3 cells, with this vector and selected with G418. We obtained no stable transfectants via this mechanism, and suspected the CMV promoter of the pEGFP plasmid resulted in production of too much topo I during selection. Topo I overexpression is known to be a problem in mammalian cells, and only insect cells have been used to produce high levels of human topo I. Consequently, we subcloned the topo I-EGFP fusion protein gene into a pIRES vector (CLONTECH), in which the topo I-EGFP and resistance cassette are under the same promoter. The production of topo I-EGFP should therefore be proportional to the selection marker used. However, again we obtained no stable transfectants in PC-3 or HeLa cell lines.

Year 2: Since we only needed topo I-EGFP as a marker, we also produced a vector containing a mutant topo I. The Stratagene Quickchange kit was used to mutate the active site tyrosine 723 into a phenylalanine residue. This produced stable, fluorescent transfectant cells but the localization of the Y723F mutant was not strictly nucleolar, indicating that this mutant would not be helpful in understanding localization of topo I.

We attempting another strategy where a knock-in/knock-out approach was used to deplete cells of native topo I with simultaneous expression of topo I-EGFP. The RNAi vector pSUPER was used to target RNAi to both the 5'-UTR and 3'-UTR of the human topo I gene. As these sequences do not exist in plasmid-borne topo I, we anticipated that selection pressure on these cells should result in expression of the topo I-EGFP chimera to replace the absent genomic topo I. However, this methodology produced no stable transfectants in prostate cancer cell lines.

Recently, a report of stable topo I-EGFP expression appeared that used a bicistronic expression vector (*1*). The authors of this paper provided us with this vector, as well as kidney cell lines expressing stable topo I-EGFP. We produced no stable transfectants with this vector either. Consequently, we abandoned the use of plasmids as a way of introducing EGFP-topo I, and instead focused on immunostaining, as detailed in Task 1.

Task 3. Measure stability of ternary complexes induced by camptothecin analogs (Months 18-30).

a. Select analogs with slowest reversal time in purified systems (months 18-24).
b. Select analogs with slowest reversal time in normal and cancer prostate cells (months 25-30).

Work toward Task 3: The proposed work on Task 3 was completed in years 1 & 2 of the project. Using a newly-developed synthetic scheme, a number of bulky, lipophilic CPT analogs were synthesized, including the 7-benzyl-CPT and 7-benzyl-MDCPT (Table 1). The newly synthesized 7-*sec*-butyl-, 7-*tert*-butyl-, 7-benzyl-, 7-benzyl-MD-, 7-*p*-tolyl-MD-, 7-*p*-chloro-MD-, and 7-*p*-fluoro-MD-CPT were characterized for their ability to inhibit topo I (EC_{50}), stabilize topo I-DNA cleavable complexes (k_{app} by competitive DNA assay), and inhibit growth of PC-3 prostate tumor cells (3-day continuous exposure MTT assay). The experimental protocols for these assays are those published previously by us (*2*). The results are recorded in Table 1. We also include previously synthesized related analogs in Table 1 for comparison.

The ability of the 7-*sec*-butyl- and 7-*tert*-butyl- analogs to inhibit topo I was comparable to CPT alone and the 7-*n*-butyl- analog, indicating a general tolerance for branched chain alkyl groups at this position. However, those analogs bearing a 7-benzyl-substituent are considerably less potent at inhibiting topo I than the analog without those substituents. Other relatively bulky groups such as 7-cyclopentyl- and 7-cyclohexyl- were effective at inhibiting topo I. However, against cultured cell lines, the growth inhibition activity of the new compounds was comparable to related analogs, and was better than CPT alone. This is most likely due to more efficient cellular accumulation of the more hydrophobic compounds.

The stability of the cleavable complexes formed with the hydrophobic 7-benzy- and 7-*tert*-butyl-CPT is similar, and these are considerably less stable than the other analogs examined. As the stability of the complex will affect the activity of these compounds in the clinical setting, the use of very hydrophobic, 7-substituted CPT as antitumor drugs will require an appropriate balance between hydrophobicity for good cellular accumulation and substituents that do not destabilize cleavable complexes. For example, the 7-*tert*-butyl-CPT

shows very poor antitumor activity *in vivo*, even compared to CPT alone. This is likely due to the poor stability of the cleavable complex formed with this analog.

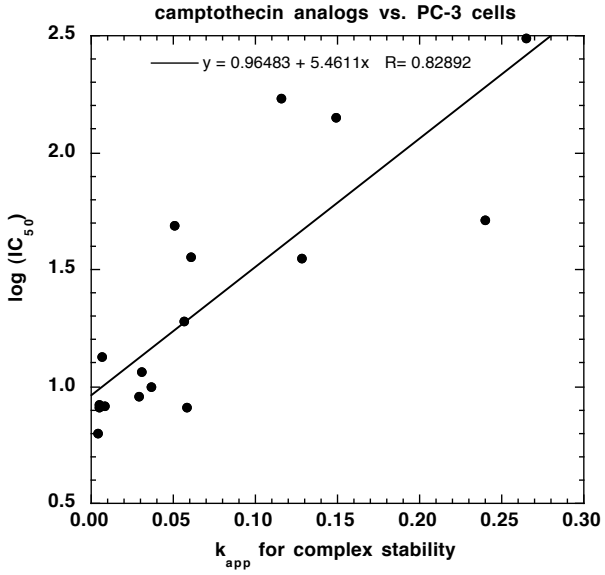
Table 1. Activity of CPT analogs with bulky 7-substituents.

CPT analog	EC_{50} for topo I (μM)	k_{app} for complex stability (min^{-1})	IC_{50} vs. PC-3 cells (nM)
CPT	0.35 ± 0.01	0.149 ± 0.010	141.4 ± 5.9
7-ethyl-10-hydroxy-CPT (SN-38)	0.32 ± 0.04	0.061 ± 0.010	35.6 ± 4.5
7-ethyl	0.07 ± 0.03	0.128 ± 0.070	35.3 ± 4.0
7-*n*-butyl	0.20 ± 0.11	0.051 ± 0.014	48.7 ± 7.2
7-*sec*-butyl	0.45 ± 0.01	0.116 ± 0.015	170.5 ± 1.9
7-*tert*-butyl	0.24 ± 0.03	0.265 ± 0.095	307.6 ± 108.3
7-benzyl	4.56 ± 3.01	0.240 ± 0.021	51.2 ± 4.7
MDCPT	0.05 ± 0.02	0.058 ± 0.003	8.1 ± 1.2
7-ethyl-MD	0.27 ± 0.05	0.037 ± 0.020	10.0 ± 1.1
7-benzyl-MD	1.95 ± 1.10	0.057 ± 0.005	18.9 ± 3.1
7-cyclopentyl-MD	0.19 ± 0.15	0.029 ± 0.008	9.0 ± 1.0
7-cyclohexyl-MD	1.0‡	0.031 ± 0.003	11.5 ± 1.8
7-phenyl-MD	0.15 ± 0.08	0.005 ± 0.001	8.1 ± 0.2
7-*p*-tolyl-MDCPT	1.04 ± 0.20	0.005 ± 0.001	8.3 ± 1.0
7-*p*-chlorophenyl-MDCPT	1.16 ± 0.10	0.008 ± 0.001	8.2 ± 0.7
7-*p*-fluorophenyl-MDCPT	0.39 ± 0.04	0.004 ± 0.001	6.3 ± 1.1
7-*p*-trifluoromethyl-phenyl-MD	0.16 ± 0.13	0.007 ± 0.003	13.4 ± 2.1

‡Inhibition (poisoning) of topo I was approximately 50 % complete at 1 μM 7-cyclohexyl-MD-CPT. However, poor aqueous solubility above this concentration prevented establishment of a complete concentration-dependent inhibition curve.

The relationship between k_{app} and IC_{50} for inhibition of PC-3 cell growth is shown graphically in Fig. 2. These data indicate that if camptothecin analogs can be developed with even more stable cleavable complexes, their activity against tumor lines will increase.

Fig. 2. Correlation of growth inhibition with k_{app} for cleavable complex stability.



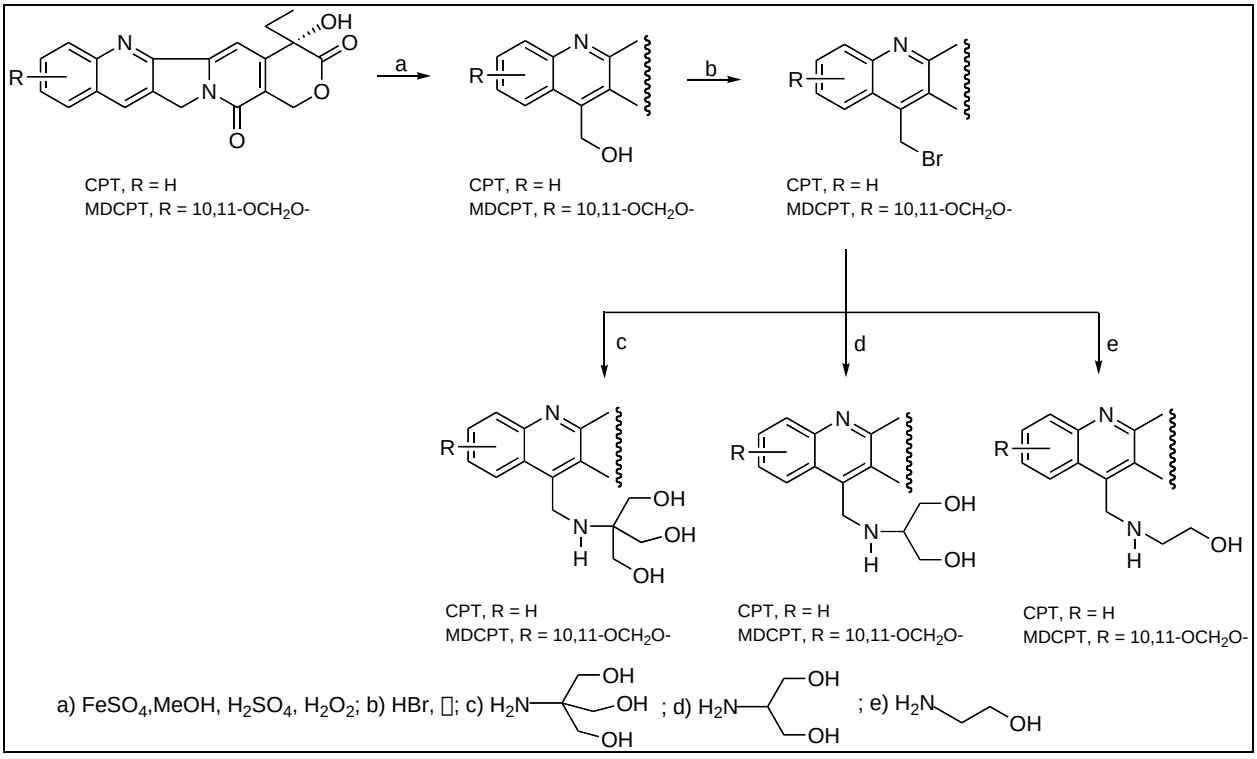


Fig. 3. Synthesis of camptothecin analogs having hydrogen bonding groups at the 7-position.

The addition of the hydrophilic groups at the 7-position did not significantly affect the ability of the CPT analogs to effectively poison topo I in assays with purified enzyme and DNA (compare MDCPT and THMAM-MD in Table 1). Hence the new compounds are very effective topo I poisons. However, one disadvantage of the increased hydrophilicity of the analogs is apparent

from their low activity in steady-state culture experiments. The THMAM analog has very low activity in inhibiting cell growth under these conditions. This is presumably due to low cellular permeation by this analog, which is approximately 2 orders of magnitude more water soluble than CPT itself (data not shown). It must be noted, however, that this type of assay is not necessarily predictive of in vivo or clinical activity of the compounds (see the data for topotecan in Table 2 for example). These data merely reflect the approximate plasma concentrations that would be necessary to begin to see antitumor activity. Hence, the THMAM-MD and related MD analogs should show activity at concentrations near or lower than those obtained with topotecan or the active metabolite of CPT-11 (SN-38).

Table 2. Activity of CPT analogs with hydrogen bonding moieties at the 7-position.

CPT analog	EC_{50} for topo I (μM)	k_{app} for complex stability (min^{-1})	IC_{50} vs. PC-3 (nM)
CPT	0.35 ± 0.01	0.149 ± 0.010	102.8 ± 7.6
SN-38	0.32 ± 0.04	0.061 ± 0.010	25.9 ± 2.1
Topotecan	7.10 ± 4.66	0.169 ± 0.009	281.7 ± 7.1
MDCPT	0.05 ± 0.02	0.058 ± 0.003	12.8 ± 1.0
7-aminomethyl-MD	0.20 ± 0.19	0.051 ± 0.002	19.7 ± 3.0
7-isopropyl-aminomethyl-MD	0.51 ± 0.02	0.047 ± 0.007	19.2 ± 4.0
7-MHMAM-MD	3.00 ± 0.86	0.029 ± 0.007	108.9 ± 40.2
7-DHMAM	4.44 ± 0.49	0.059 ± 0.007	807.3 ± 128.8
7-DHMAM-MD	0.04 ± 0.02	0.023 ± 0.002	206.8 ± 31.5
7-THMAM	2.82 ± 1.86	0.037 ± 0.013	2078.2 ± 405.4
7-THMAM-MD	0.11 ± 0.07	0.006 ± 0.001	90.8 ± 24.5

Task 4. Measure induction of apoptosis in normal and cancer prostate cells (Months 31-36)

a. compare induction of apoptosis in rapidly dividing and slow-growing normal and cancer cells (months 31-35)
b. Correlate apoptosis data with data from tasks 1 and 3 (month 36)

Work toward Task 4.

As Task 4 required input from Tasks 1-3, we did not begin work on this Task. In the Approved Statement of Work, this Task was to begin during the final year of funding. We requested a 6 month no-cost extension to our grant period within which time we would use the remaining funds for this project (approximately $10K in direct costs) to attempt apoptosis measurements on PC-3 cancer cells using a caspase 3 activation assay..

KEY RESEARCH ACCOMPLISHMENTS:

- Evaluation of camptothecin analogs for activity against prostate cancer cells.
- Development of camptothecin analogs that form stable cleavable complexes.

REPORTABLE OUTCOMES:

- Manuscript in preparation for submission to *Bioorganic and Medicinal Chemistry Letters*. To be submitted in 2006.

CONCLUSIONS:

In this final report, the data generated during 2002-06 is presented, and described in the context of the Approved Statement of Work. We have identified a highly active camptothecin analogs that is active against PC-3 prostate cancer cells. This may be studied in animal models if future grant proposals are funded. If a no-cost extension for 6 months is approved, we will use the remaining funds to accomplish as much of task 4 as possible in that time-frame.

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